The effects of DLL3 in axial skeletal development and glioma

A Senior Honors Thesis

Presented in partial fulfillment of the requirements for Graduation with Research Distinction in the School of Health and Rehabilitation Sciences at The Ohio State University

Rachel D'Amico

April 7, 2014

Advisor: Susan E. Cole, PhD, Department of Molecular Genetics

Table of Contents

Abstract

The Notch pathway is critical for normal development of the skeleton and nervous system. Dysregulation of Notch can also promote glioma, cancer of the glial cells. Tight regulation of Notch is achieved by modulating pathway activity through inhibitory ligands (like DLL3) and/or glycosyltransferases that affect receptor-ligand interactions (like Lunatic Fringe). In most contexts, increased *Lfng* causes an increase in Notch activity, while *Dll3* expression causes a decrease in Notch activity. Our project looked at this modulation in two systems: axial skeletal development and glioma cells. In the presomitic mesoderm (PSM), loss of *Lfng* increases Notch activity while a loss of *Dll3* causes a decrease in Notch activity. Examination of skeletal phenotypes and Notch activation patterns in double knockout mice shows that the *Dll3*-null phenotype is epistatic to the *Lfng*-null phenotype in the posterior PSM, suggesting that in a molecular clock-dependent setting, LFNG acts through DLL3 to inhibit Notch activation. However, in the anterior PSM, LFNG and DLL3 work through different pathways to regulate Notch. In contrast, in glioma Notch signaling maintains glioma stem cells, which worsen outcomes. Increased *Dll3* expression in glioma tumors leads to better prognosis, suggesting DLL3 may inhibit Notch activity. To study this hypothesis, we used Luciferase assays in glioma cells stably transfected with a luciferase gene under the control of a Notch-dependent promoter, as well as MTS viability assays. We found that increasing *Dll3* expression decreases Notch signaling, but does not affect cell viability (a Notch-independent phenotype). DLL3 appears to be a promising inhibitor of Notch activity in glioma cells, suggesting it could be targeted in future treatment options. We have found that regulation of Notch is highly context dependent, both in development and disease.

Table of Figures

Introduction

I. The Notch pathway

The Notch signaling pathway is highly conserved in vertebrates, and is essential to controlling cell fate through differentiation. In some cases, it helps to maintain stem cell populations, and once the cells are ready to differentiate, promotes one cell type over another, thus ensuring not all cells commit to the same fate (Bray, 2006). When mutated, dysregulated Notch signaling is known to have pathogenic effects. Mutations in members of the Notch pathway lead to Alagille syndrome, spondylocostal dysostosis, and cerebral arteriopathy (reviewed in Gridley, 2003). Further, dysregulated Notch activity has been detected in many different cancers (Koch and Radtke, 2007).

A. *Notch has a complex mechanism of action*

Notch activity is controlled by its interactions with DSL (Delta, Serrate, Lag2) ligands. The Notch receptor is synthesized as a polypeptide, then displayed as a transmembrane protein, with multiple epidermal growth factor (EGF)-like repeats available for ligand binding. When interaction occurs with a DSL ligand on a neighboring cell, the extracellular domain of Notch is cleaved by ADAM, a metalloprotease. The Notch intracellular domain (NICD) is then cleaved from the membrane-spanning region by γ-secretase (encoded by Presenilins 1 and 2) on the inside of the cell membrane. NICD translocates to the nucleus, where it binds to the transcription factor CBF-1. CBF-1 then turns the CSL complex from a transcription repressor to an activator, and CSL activates Notch's target genes, like the *Hes* family, which regulate transcription and prevent neural differentiation gene expression (as reviewed by D'Souza, 2010).

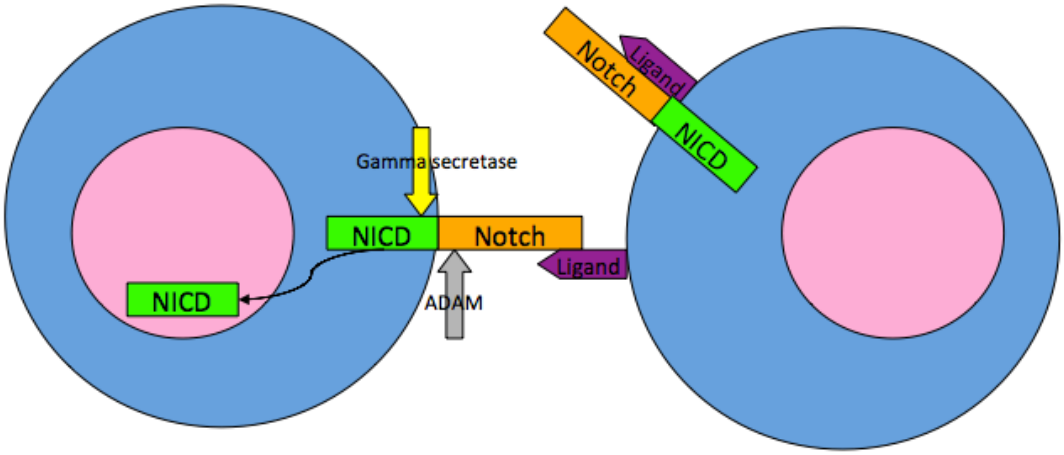

Figure 1. Notch receptors undergo trans-activation/cis-inactivation with DSL ligands. When a Notch receptor and a ligand are expressed on neighboring cells, the proteins bind together. ADAM metalloprotease releases the extracellular portion of the receptor while gamma-secretase cleaves Notch intracellularly. This produces the Notch Intracellular Domain, or NICD, which translocates to the nucleus and controls transcription of its target genes. If the Notch receptor is expressed in the same cell as the ligand, the ligand inhibits binding to an extracellular ligand, and thus prevents the release of the NICD fragment and activation of the pathway.

A unique feature of the Notch pathway is ligand trans-activation/cis-inactivation. If Notch is expressed in a cell next to a ligand-expressing cell, then the ligand is able to initiate cleavage of Notch and activation of its pathway. But when a ligand is expressed in the same cell as Notch, NICD release and action can be inhibited (Bray, 2006). This most likely evolved as a way to provide regulation of the pathway, preventing all cells from entering the same cell fate, and to direct tissue patterning in a polarizing way. No two neighboring cells will both express activated Notch, ensuring ample tissue variation.

B. Notch signaling is upregulated in many cancers

The Notch pathway has been shown to be overexpressed in leukemia, medulloblastoma, melanoma, breast, pancreatic, and colorectal cancers. Two thirds of ductal carcinoma tumors were found to be positive for Notch-1 staining and half of all breast cancer tumors had loss of Numb expression, a negative regulator of the Notch pathway, indicating Notch's widespread effects and importance in cancer. Interestingly, while in most cases it is oncogenic, Notch-1 is

capable of also acting as a tumor suppressor. Animals with downregulated Notch-1 expression in skin cells develop basal cell tumors (most likely due to its role in Shh and Wnt signaling), and Notch-1 deficient skin is more susceptible to cancer. The Notch pathway is also important as a tumor suppressor in hepatocellular, skin, and small cell lung cancers (reviewed in Koch and Radtke, 2007). These contrasting roles are reminiscent of Notch's ability to either promote progenitor stages or induce differentiation depending on the tissue.

Notch signaling was first shown to be of importance in glioma in 2002, when it was found that human glioma cells preferentially overexpress Jagged1 and DLL1, both Notch ligands (Ignatova et al., 2002). Inactive Notch signaling is one of the defining characteristics of low-grade glioma, and has been experimentally shown to be significant in outcome. When Notch-1 activity is repressed, it causes decreased proliferation, increased apoptosis, and decreased tumorigenic capabilities (Purow et al., 2005). Conversely, overexpressing Notch-1 in glioma cells increases their growth rates and colony formation, as well as stem cell expression markers like Nestin (Zhang et al., 2008).

Recently, a phase I study tested the γ-secretase inhibitor (which prevents the release of the activated NICD fragment) MK-0752 in patients with solid tumors. The most significant response was observed in patients with central nervous system cancers. Ten out of the twelve patients that exhibited prolonged stable disease had glioma, and one patient with anaplastic (grade III) astrocytoma exhibited a complete response for over a year (Krop et al., 2012). This demonstrates the drug's ability to cross the blood-brain barrier and navigate the extracellular matrix, both of which are of major concern in glioma treatment. However, there are some drawbacks to γ-secretase inhibitor treatment, including side effects due to Notch and γ-secretase's activity throughout the body.

II. Somitogenesis and the segmentation clock

Somitogenesis is the developmental process that allows somites, which eventually develop into vertebrae, ribs, and skeletal musculature, to bud off of the presomitic mesoderm (PSM) in vertebrates, located at the distal end of the embryo. This highly regulated process is very methodical, occurring every 2 hours in mice, and continues even when the PSM is removed from the rest of the embryo and left in culture (Christ et al., 1974). It has been suggested that a molecular clock, or the cyclical expression of regulatory genes, provides the necessary temporal coordination.

The current understanding of segmentation is the clock and wavefront model. This model suggests that a cell oscillates between permissive and non-permissive states ("the clock"), and cells that are close to one another will go through the same state at the same time, thus creating a wave of gene expression that passes through the PSM. The period of this oscillation is the same as the time between the formation of new somites. As the clock continues, the wavefront moves down the PSM caudally, and when cells in the permissive state pass this wavefront, they begin to bud off into somites as organized cohorts of cells (Cooke and Zeeman, 1976). Molecular evidence for this model was provided when the gene *c-hairy1* was shown to cause segmentation in a manner that was predicted by the clock model (Palmeirim et al., 1997).

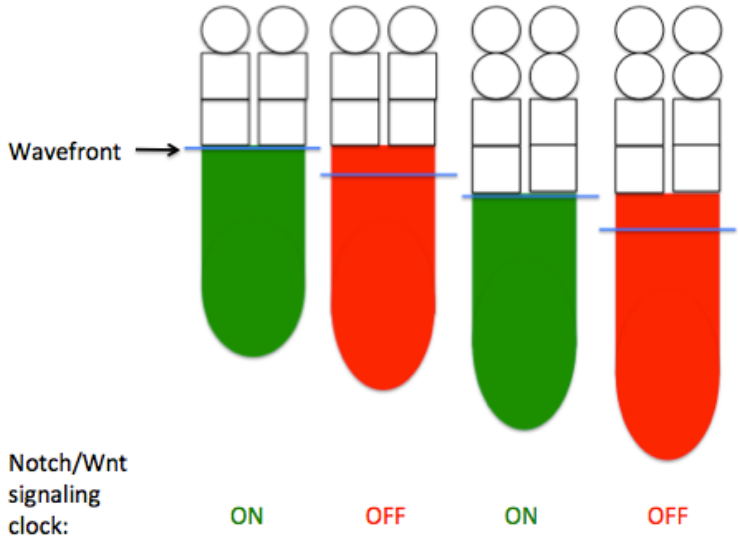

Figure 2. The clock and wavefront model showing the posterior undetermined region of the PSM, with the anterior region forming pre-somites and somites. The Notch and Wnt pathways are responsible for the oscillating clock, where gene expression changes at a timed rate. As the wavefront, created by the FGF pathway, meets cells in a permissive state, they start to segment off into presomites.

The Notch pathway is critical for controlling the clock. Activation of the Notch pathway initiates expression of *Hes7*, which has been shown to be one of the most important genes for segmentation; knocking out or constantly expressing *Hes7* causes severely fused vertebrae and ribs (Bessho et al., 2001, and Hirata et al., 2004). It is the oscillation of *Hes7*, as part of the clock, that allows for normal segmentation. There are multiple ways that *Hes7* expression oscillates; first, *Hes7* forms a negative feedback loop, and represses its own expression by binding to its promoter (Bessho et al., 2001). Cyclical activation of Notch also affects *Hes7* expression, and this is achieved by changing expression of its activating modulators, especially Lunatic Fringe.

Lunatic Fringe (or LFNG) is a glycosyltransferase, that glycosylates the Notch receptor to modulate its affinity to bind to other ligands and become activated (Bruckner et al., 2000). LFNG activity also fluctuates in the PSM, and constant expression or loss of expression of *Lfng* causes

somite fusion (Evrard et al., 1998). It has been shown that *Hes7* negatively regulates LFNG as well as itself; when *Hes7* is repressed, *Lfng* is expressed ubiquitously, whereas when degradation of *Hes7* is inhibited, *Lfng* is repressed (Bessho et al., 2001). However, it has been found that in the PSM, repression of *Lfng* causes an increase of Notch activity, suggesting that in this context, LFNG modulation inhibits the pathway (Shifley et al., 2008).

III. Glioma

Glioma is the cancer of the glial cells in the central nervous system, and is the most common primary brain malignancy. The most aggressive form, glioblastoma multiforme (GBM), has a predicted survival of 12 months with tumor resection, chemotherapy, and radiation (Kanu et al., 2009). This unfortunate prognosis is largely due to their unique phenotype: GBMs are extremely heterogeneous, highly invasive and unusually resistant to chemotherapeutic drugs, and there is insufficient understanding of these mechanisms (Wen and Kesari, 2008). The current standard of care for glioma, temozolomide (TMZ), is an alkylating agent that causes double-stranded breaks in DNA. However, 40% of patients undergoing chemotherapy do not show any response to TMZ therapy (Mrugala, 2008); this is partly due to the fact that the cell has a natural way to combat this alkylation, using the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). This protein removes alkyl groups, inhibiting the effect of TMZ. It has been shown that methylation (and thus inactivation) of the MGMT promoter in tumor cells is correlated with better response to TMZ and better survival, with these patients living an average of 9 more months than those patients with unmethylated MGMT promoters (Hegi et al., 2005). TMZ has also been found to cause secondary cancers, specifically leukemia and skin cancers (Sanderson and Shield, 1996), but few other therapeutic options have been successful.

Glioma stem cells (GSCs) have been suggested as a source of the glioma tumor and contributors to its characteristic invasive nature and high recurrence rate (Wen and Kesari 2008). They are thought to be related to "neural stem cells," progenitor cells in the nervous system that are multipotent and are capable of self-renewal, and are responsible for neurogenesis and gliogenesis in the fetal brain. GSCs are most commonly identified by expression of CD133, a cell surface protein that is characteristic of neural stem cells, however it has been noted that probably not all CD133+ cells in a tumor are GSCs (Beier et al., 2008). GSCs are capable of multipotency, telomerase activity, self-renewing capabilities, and resistance to apoptosis (as reviewed by Dell'Albani, 2008).

Higher-grade gliomas are often more undifferentiated, suggesting that an increased number of GSCs worsen outcome (Phillips et al., 2006). GSCs have been found to grow in neurospheres in culture, or small, floating spheres of cells all derived from the same original cell. The outside layers of these spheres are heterogeneous, expressing both neuronal and glial markers, but the inner core of cells express stem cell markers (Vik-Mo et al., 2011), illustrating GSC cells' ability to create a diverse tumor, one of the major treatment challenges in glioma. CD133 expression increases dramatically in recurring gliomas as opposed to their primary tumor (Liu et al., 2006), suggesting that GSCs are associated with tumor recurrence and chemoresistance.

GSCs are relatively quiescent, and their slow replication means that they are not as affected by alkylating drugs as other tumor cells, allowing them to survive with no or relatively few DNA breaks until treatment is over. CD133+ cells also have higher expression of the MGMT protein than CD133- cells in the same tumor, meaning they are able to avoid the effects of TMZ (Liu et al., 2006); this could be partially responsible for the glioma's lack of response to treatment. All

of this implies that GSCs may be one of the major problems in glioma, and to treat the disease we need to target these initiating cells.

The Notch pathway is upregulated in undifferentiated embryonic CNS cells, and is hypothesized to inhibit differentiation (Lasky and Wu, 2005); because of this, it has become of new interest in glioma. Notch activation in glioma cells has been shown to enhance DNA repair through the Akt pathway, promote cell growth and tumor formation (reviewed in Natsume et al., 2011), and its upregulation is considered a hallmark of GSCs. Notch expression has been shown to induce a GSC phenotype: when the NICD fragment of the Notch receptor was stably transfected into a glioma line, it was found to have higher colony- and sphere-forming capabilities, both markers of GSCs (Zhang et al., 2008). But inhibition of Notch-1 has proven deadly to GSCs. When Notch activity was inhibited by γ-secretase inhibitors, the CD133+ population was decreased 5-fold. While more differentiated cells' growth was unaffected by Notch activity inhibition, their ability to form colonies and tumor xenografts was suppressed, suggesting that the cells that initiate and propagate the glioma were gone (Fan et al., 2006).

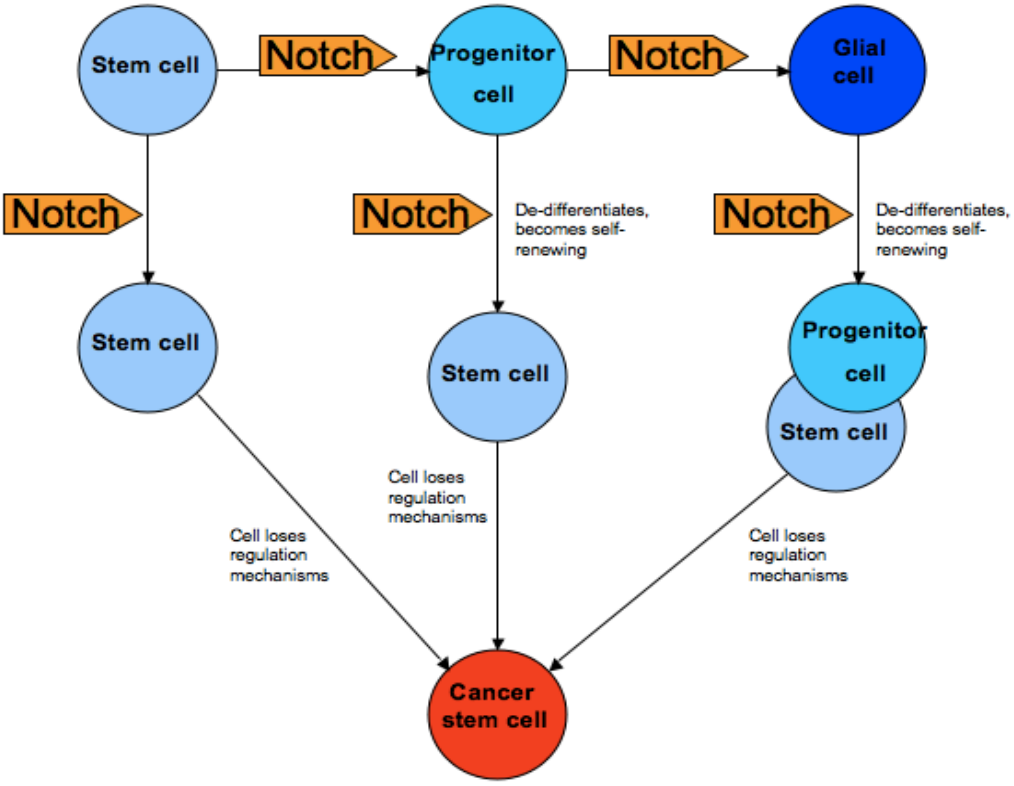

Figure 3. The role of the Notch pathway in glioma stem cell creation. Normally, Notch maintains normal neural stem cells and pushes progenitor cells towards a glial cell fate. However, Notch can also cause de-differentiation in the cell; this causes the cell to become self-renewing, increase DNA repair ability, and become quiescent. While Notch is not the only mechanism involved in GSC creation (for example, other pathways must be mutated to change regulation of cell replication), it is the beginning of a series of pathogenic mutations leading to cancer stem cells (adapted from Goldthwaite, 2006).

Drug targets for GSCs are essential if the prognosis of GBM is to be improved and the recurrence of glioma is to be reduced. If GSCs are responsible for glioma recurrence and are unresponsive to TMZ, then we are not treating the source of the problem. As the current standard of care, TMZ only prolongs the disease without eradicating the tumor source, but unfortunately, it is currently the best treatment we have. GSCs' quiescent nature makes current therapies less effective, as many cancer treatments require the cells to be actively replicating. Most targets of current interest would cause the GSCs to differentiate and begin to replicate more quickly, making them susceptible to conventional treatment. These targets include the Notch, Wnt, and

Hedgehog pathways, which are all required for stem cell growth. By utilizing our knowledge of normal neural stem cells and cell differentiation in the brain, we can begin to target GSCs.

IV. DLL-3

The most structurally divergent Notch ligand is DLL3; it is considered to have a degenerate DSL domain, the segment that interacts with the Notch receptor and is a commonality between all other Notch ligands. It also lacks the DOS (Delta and OSM-11) motif and intracellular lysine residues, which are both considered central to trans-activation (as reviewed in D'Souza, 2010). DLL3 has been shown to lack the ability to trans-activate Notch-1 in normal development, but when expressed in the same cell as Notch it can cis-inhibit (Geffers et al., 2007; Ladi et al., 2005). DLL3 has been shown incapable of replacing DLL1, a ligand that can both activate and inhibit the Notch pathway, (Geffers et al., 2007)

It has been proposed that DLL3 targets Notch-1 for lysosomal degradation, therefore preventing cell membrane presentation and sequestering the ligand in the Golgi (Geffers et al., 2007), although other authors have found that DLL3 bound to Notch-1 does not prevent its exposure in the membrane (Ladi et al., 2005). In addition to these conflicting explanations, the subcellular expression of *Dll3* has never been analyzed in tumor cells, underscoring the need to further understand the molecular mechanisms of this protein in cancer.

Importantly, *Dll3* expression was found to be higher in proneural GBM samples that had better prognoses. These tumors did not have upregulated Notch-1, suggesting that its Notch-inhibiting properties could naturally decrease malignancy (Phillips et al., 2006). Fibulin-3 is a protein normally not present in the brain, but has been found to be upregulated in gliomas. It has been shown to be an activator of Notch signaling, and promotes invasion and tumor survival. Downregulation of Fibulin-3 caused apopotosis, and impaired the growth of intracranially-

implanted tumors. DLL3 has been shown to antagonize and coprecipitate with Fibulin-3, effectively inhibiting its activation of the Notch signaling pathway (Hu et al., 2012). However, DLL3 has never been purposefully manipulated to study its effects on Notch-1 activity and resulting tumor phenotype.

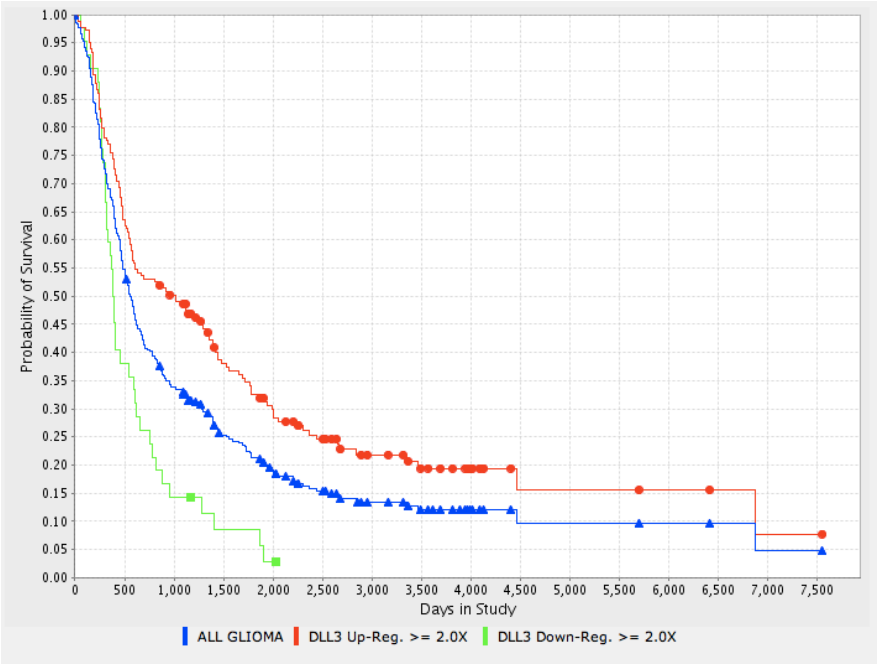

Figure 4. Kaplan-Meier survival plot of gliomas with different *Dll3* expression levels. Downregulated *Dll3* (2x below the average *Dll3* expression) has a worse outcome than all glioma tumors (p-value= 0.002), while upregulated *Dll3* (2x above the average expression) has a better prognosis (p-value<0.001). This suggests that DLL3 is involved in promoting a more treatable tumor, possibly through the Notch pathway. Data from REMBRANDT (National Cancer Institute).

DLL3 usually inhibits Notch activity, which would explain why increased expression of *Dll3* prolongs survival. However, Notch modulation is very context dependent; loss of *Dll3* in the PSM decreases Notch activity suggesting that in that context, DLL3 is involved in Notch activation. Thus, our objectives focused on starting to understand the function of DLL3 in a context where it activates Notch and examine the role of DLL3 in the Notch pathway in glioma cells. By understanding the role DLL3 plays in two different contexts, we can begin to

understand if DLL3 could present a unique opportunity as an endogenous inhibitor of Notch signaling that could be used to develop targeted therapies against glioma.

Objectives

Specific Aim 1: What is the epistatic relationship between Lunatic Fringe and DLL3?

Based on previous phenotypic findings in mice, we hypothesize that DLL3 will be epistatic to LFNG. By understanding the relationship between these two modulators, we can understand the function of DLL3 in normal development, its ability to control the Notch pathway, and its potential as a glioma treatment target.

Specific Aim 2: What are the effects of overexpressing *Dll3* in glioma cells?

We hypothesize that *Dll3* overexpression will decrease Notch activity and will affect Notch-dependent phenotypes, like chemoresistance, but will not change Notch-independent phenotypes, like viability, because of DLL3's specificity to the Notch pathway.

Materials and Methods

Mice strains and upkeep

Lunatic Fringe null ($Lfng^{tmRj01}$) and DLL3 pudgy ($DLL3^{pu}$) mice have previously been described (Evrard et al., 1998; and Kusumi et al., 1998). Mice were maintained on mixed 1295v/C57BL6/GVB backgrounds by intercrosses of $DLL3^{pu/+}$; $Lfng^{+/-}$ mice. For timed pregnancies, cages were checked daily with noon of the day of plug detection defined as 0.5 d.p.c. (days post coitum). All procedures were conducted under protocols approved by the OSU IACUC.

Mice and embryo genotyping was completed using tail preps and yolk sacs, respectively. For tail DNA analysis, samples were processed overnight with ProteinaseK, then DNA was salted out using sodium chloride and washed with ethanol. Yolk sac DNA was prepared via the HotSHOT procedure (Truett et al., 2000). PCR genotyping used primers SC355 (5'-GCCTCTTCTTCAGGGTCTGC-3') and SC356 (5'-ACTCACCGGCCAAGCATC-3') for *Dll3*; and FNG322 (5'-GAGCACCAGGAGACAAGCC-3'), FNG325 (5'-AGAGTTCCTGAAGCGAGAG-3'), and PGK3 (5'-CTTGTGTAGCGCCAAGTGC-3') for *Lfng*. The *Dll3* PCR products were digested with HaeIII (from Biolabs) for 2 hours at 37° C. PCR/digest products were run on a 2.5% agarose gel and analyzed by comparison to controls. *Lfng* PCR produced bands at 410 bp ($Lfng^{-/-}$), 380 bp ($Lfng^{+/+}$), or both ($Lfng^{+/-}$). *Dll3*PCR and digest produced bands at 1000 bp ($Dll3^{+/+}$), 1300 bp ($Dll3^{p/p}$), or both ($Dll3^{+/p}$).

Whole mount immunohistochemistry

10.5 d.p.c. mouse embryos were dissected and fixed in 4% PFA overnight. Embryos were washed in methanol and bleached overnight. After washing in PBS and CT, embryos were heated and incubated in primary antibody against NICD (Cell Signaling) at 1:300 dilution for 5

days at 4° C. Embryos were washed in MABT and incubated overnight with AP-tagged anti-rabbit IgG secondary antibody (Cell Signaling cat. #4147) at a 1:500 dilution. After exposure to the antibodies, embryos were washed with MABT+2 mM Levamisole overnight. Detection was performed in detection solution, and the embryos were checked every 30 minutes until desired staining appeared. Embryos were washed, dehydrated with methanol, and rehydrated. Embryos were then cleared in 1:1 Glycerol:PBT and 4:1 Glycerol:PBT.

Skeletal Preparations

17.5 d.p.c. mouse fetuses were skinned and fixed in 95% ethanol for five days. The fat was removed by placing the fetuses in acetone for 2 days. They were stained for 3 days at 37° C in 0.015% alcian blue and 0.005% alizarin red. After staining, the fetuses were washed with distilled water and cleared in 1% KOH in 20% glycerol as needed, with progress being monitored every 3-4 days. They were then moved into 50%, 80%, and 100% glycerol for storage.

Cell culture and expression constructs

The major cell line to be used for this project was the rat glioblastoma cell line CNS-1. These cells share many properties of invasive human glioma with neural progenitor-like characteristics, including nuclear atypia, pleomorphism, and expression of glial and neuronal proteins, including Notch (Kruse et al., 1994). Cell lines were obtained from Mariano Viapiano. RPMI media with 10% FBS was used for culture, with media being changed every 4 days; cells were passed using 0.05% Trypsin as needed.

Overexpression of *Dll3* was achieved by transient transfection with JetPrime transfection reagent with a full-length rat *Dll3* clone carried in the vector pcDNA4.1/V5x6His.

Quantitative RT-PCR

To observe endogenous *Dll3* transcription, qRT-PCR was performed. RNA was isolated from CNS-1 cells or rat brain, and underwent reverse transcription into cDNA (Superscript RT, Invitrogen). Quantitative PCR with the obtained cDNA using SYBR Green was then performed with primers for *Dll3* (5'-TTTCCAAGGCTCTAACTGTG-3' and 5'-AGGTCGTGCTCGCAGCGT-3') and GAPDH (5'-AGTCCATGCCATCACTGCCAC-3' and 5'-ATGACCTTGCCCACAGCCTTG-3'). GAPDH is an enzyme involved in glycolysis and is expressed in every cell; it is therefore used to determine the amount of cDNA present. *Dll3* expression was normalized using GAPDH.

Luciferase co-culture assays

Cells were counted and plated (40,000 CNS-1 cells per well in 24-well plate and 200,000 per well in 6-well plate). After 24 hours, the cells in the 24-well plate were transiently transfected with 100 ng of JH-26 (CBF-controlled Luciferase), 100 ng pBOS-Notch1, 10 ng renilla (transfection control), and 500 ng of an interest gene (either pcDNA control or *Dll3*). Cells in the 6-well plate were transiently transfected with either 2 μg of pBOS or *Dll1* expression vector. After 24 hours, 15,000 cells from either the pBOS or *Dll1* wells were placed in each of the 24-well plates. After 24 hours, cells were lysed with Passive Lysis Buffer and luciferase and renilla levels were measured with a luminometer and a Promega Luciferase Kit. To control for varying transfection efficiencies, luciferase activity was normalized to renilla activity (L/R). Experiments were performed in triplicate.

MTS viability assays

Cells were transfected with either a pcDNA control vector or *Dll3,* then counted and plated (5,000 CNS-1 cells per well in a 96-well plate). Cells were left to grow for 2-7 days. At

different time points, MTS reagent (from the Promega CellTiter kit) was added and the plate was incubated until sufficient coloration had appeared. MTS is reduced in metabolically active cells into formazan. The absorbance of formazan at 490nm can be measured, and is directly proportional to the number of active cells. Numerical data was derived from a plate reader, which measured absorbance at a wavelength of 490 nm. Experiments were performed in triplicate.

Analysis of chemoresistance

To examine the effect of DLL3 on cells' resistance to chemotherapy, cells were transfected with the pcDNA-*Dll3* construct or an inert control plasmid, and 24 hours later exposed to 400 micromolar temozolomide (TMZ), the standard of care chemotherapeutic drug for glioma, or isovolumetric DMSO for varying time points. Cells were assessed by MTS redox assays to determine if changes in DLL3 activity affected the cells' ability to survive treatment.

Statistical Analysis

Grouped results were analyzed by t-tests or one/two-way ANOVA according to the experimental design. All differences were deemed significant at $p<0.05$.

Results Pertaining to Segmentation

The first objective of this thesis was to study the interaction between the Notch modulators DLL3 and Lunatic Fringe. We hypothesized that *Dll3* would be epistatic to *Lfng* because of previous results based on phenotype, however the relationship between the two modulators has not been studied on a molecular basis.

To achieve this, we used a cross of mice that have *Lfng* knocked out (Evrard et al., 1998) and those with a dysfunctional *Dll3* allele, called pudgy (Kusumi et al., 1998). By using mice that have nonfunctional *Lfng* and *Dll3*, we were able to see which gene controlled the phenotype of the mice that were homozygous for both mutations (also known as "double mutants").

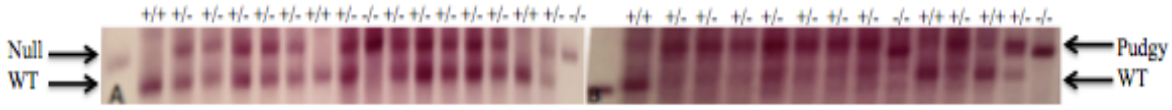

Figure 5. *Lfng* PCR genotyping results (Image A) show homozygote wildtype embryos (lanes 1, 7, and 15), heterozygotes (lanes 2-6, 8, 10-14, 16), and homozygote null embryos (lane 9); these were compared to a *Lfng* null control in lane 17. *Dll3* genotyping (Image B) show homozygote wildtype (lanes 1, 10, 12), heterozygotes (lanes 2-8, 11), and homozygote pudgy (lane 9); these were compared to a heterozygote (lane 13) and homozygote pudgy (lane 14) control. Heterozygotes of both Lfng and DLL-3 were classified as wildtype, as both mutations are loss-of-function.

Double mutant embryos show DLL-3 mutant Notch activation pattern

To observe how Notch activation changes in double mutants, 10.5 d.p.c. mouse embryos were probed for NICD, the activated form of Notch-1, in the presomitic mesoderm (PSM) by whole mount immunohistochemistry using an antibody that is specific for the cleaved NICD fragment. Instead of studying the basic expression of Notch, this shows where the pathway has been activated, and where expression of target genes would be expected. The modulators (including LFNG and DLL3) do not control the concentration of the Notch receptor, only the activation of it, so this allows us to study their role in affecting the Notch pathway.

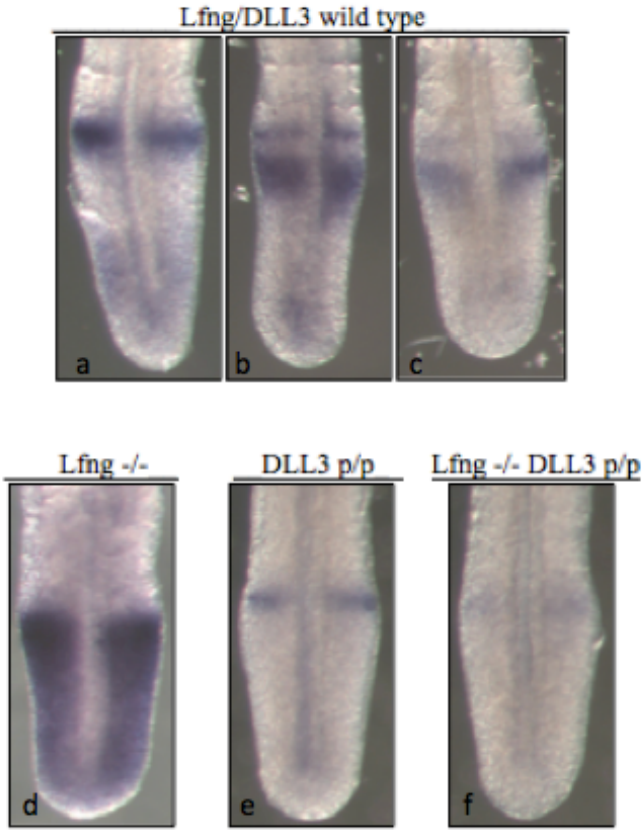

Figure 6. Wildtype (a-c, n=6), *Lfng*-null (d, n=4) *Dll3*-pudgy (e, n=5), and double-mutant (f, n=4) presomitic mesoderms of 10.5 d.p.c. mouse embryos. The embryos with both *Lfng* and *Dll3* knocked out have a similar Notch activation pattern to those of *Dll3*-pudgy embryos in the posterior PSM, in that both show decreased Notch activation in comparison with the wildtype and *Lfng*-null embryos. However, in the anterior PSM the double mutants' phenotype appears more severely affected than the *Dll3*-pudgy embryos, as their Notch activation bands are fainter.

In the wildtype embryos, the bands of activation oscillate, as expected; since each embryo is caught in a different stage of the cycle, we observe several distinct patterns of activation. In embryos with *Lfng* knocked out, Notch activity is visible throughout the whole PSM, running counter to what we know of the LFNG/Notch relationship. When an activator of Notch (like LFNG) is knocked out we would expect a decrease in activity; thus it seems that in the PSM, LFNG is involved in Notch pathway inhibition. When *Dll3*, which is usually considered an inhibitor of Notch, is knocked down, Notch activity decreases when we expected increased activity of Notch. This suggests that the associations between Notch and its modulators

are much more complex than we currently understand. As such, it is important that more research is done to establish the complex associations in this pathway.

In embryos with both *Lfng* and *Dll3* knocked out, the embryos show a similar expression pattern to *Dll3* knockouts in the posterior PSM, however they are more severely affected in the anterior PSM (which is not regulated by the clock) as the bands appear fainter. In the clock, which controls the posterior PSM, the *Dll3* phenotype is epistatic to *Lfng*, suggesting that LFNG acts on Notch in a way that requires DLL3 activity. In the anterior PSM, which is clock-independent, the phenotypes seem additive, suggesting that *Lfng* and *Dll3* act in parallel pathways.

Double mutant mouse skeletons show worse phenotypes

The regular action of both DLL3 and LFNG as modulators of the Notch pathway is crucial to normal skeletal development, however the two have rarely been studied together. To study the effect of both mutations on axial skeletal phenotype, 17.5 d.p.c. mouse fetus skeletons were selectively stained for bone and cartilage using alizarin red and alcian blue, respectively. Regulation of Notch signaling is imperative for normal axial skeleton development; by studying mice with both mutations, we will be able to assess which gene is epistatic to the other and controls the ultimate phenotype of the mouse.

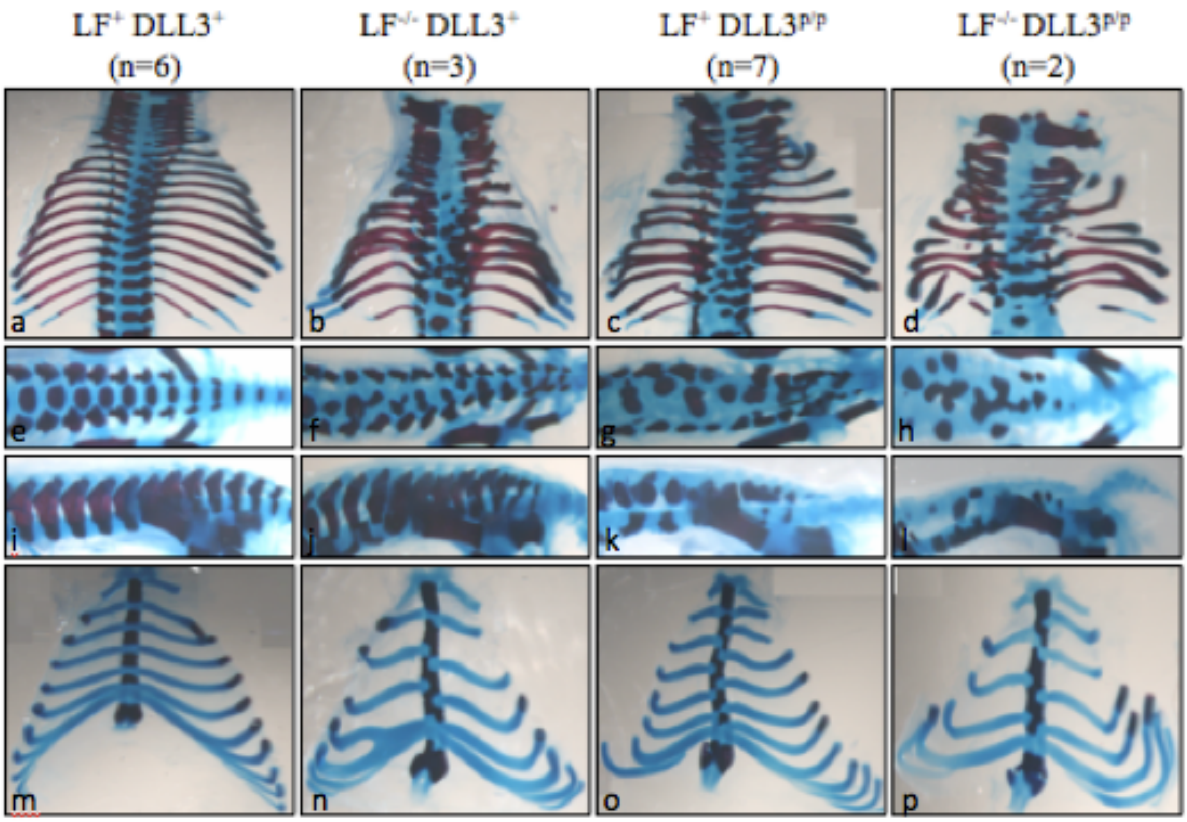

Figure 7. Skeletal preparations of 17.5 d.p.c. mouse fetuses of indicated genotypes were performed using alizarin red to stain bone and alcian blue to stain cartilage. Views of the ribs, both dorsal (a-d) and ventral (m-p) are shown, as well as a dorsal view of the sacral region (e-h) and a coronal view of the sacral region (i-l).

Mutations in either *Lfng* or *Dll3* alone causes disorganization in the ribs and spine, including offsetting of the ventral ribs' pairings, bifurcations and unattachments of the dorsal ribs, and unorganized patterning of the vertebrae. There is more visible rib disorganization in the double mutants' ribs than either the *Lfng*-nulls or the *Dll3*-pudgy skeletons, as seen in the dorsal view of the ribs, however from the ventral view the ribs' cartilage does not appear more affected. There is no rescuing of the double mutants' phenotype in the posterior vertebrae, as there is in *Lfng*-nulls, suggesting that in the posterior, *Dll3* is epistatic to *Lfng*. We also observed that double mutants lack organized neural arches (as visible in the coronal view of the sacral region), which are present in

both *Lfng*-nulls and *Dll3*-pudgy mice. This suggests that the formation of neural arches is dependent on the presence of one of these genes, and that a nonfunctional *Lfng* or *Dll3* can be rescued in the neural arches by the action of the other.

These results indicate that there are different relationships between DLL3 and LFNG in the anterior and posterior PSM, which then manifest themselves in the skeletal phenotype. There is no clear epistatic relationship between *Dll3* and *Lfng* in the anterior PSM. It appears that DLL3 and LFNG act together in the controlling of the Notch pathway through parallel systems, which causes a decrease in Notch activation in 10.5 d.p.c. double mutant embryos' anterior PSMs, and a worsening of rib abnormalities in 17.5 d.p.c. skeletons. We also found that the two genes interact in previously undescribed ways to form neural arches, suggesting that one of these two parallel systems is required for the development of neural arches. In the posterior PSM, *Dll3* is epistatic to *Lfng*, which prevents the clock from organizing the developing somites. We may speculate that this is why in the posterior vertebrae, there is no rescuing of the double mutants' phenotype as there is in *Lfng*-null mice. While we see that in the context of the clock, DLL3 is required for LFNG activity, in a clock-independent situation they function collaboratively. This data shows us that even in the single process of segmentation, the effects of DLL3 and other modulators, like LFNG, can have distinct effects on Notch signaling in different contexts. This makes it critically important to understand exactly how Notch is being regulated in gliomas before we begin to target it in therapy.

Results Pertaining to Glioma

The second aim of this project was to study the effect of overexpressing *Dll3* in glioma cells. As previously shown, upregulated *Dll3* betters survival of glioma, however its role is not clear. We hypothesized that an overexpression of *Dll3* would decrease Notch activity, and tested this by Luciferase assay. We also wanted to observe whether the overexpression of *Dll3* would cause phenotypic changes, so both Notch-independent (cell viability) and Notch–dependent (chemoresistance) phenotypes were tested.

DLL3 is underexpressed in CNS-1 cells compared to normal rat brain tissue

In the interest of familiarizing ourselves with the relative expression of *Dll3* in our main cell line, gel electrophoresis and quantitative RT-PCR were performed on normal rat brain and CNS-1 cells.

We find that CNS-1 cells have extremely low DLL3 expression compared to both rat brain and rat embryo. Qualitatively, Western blot analysis showed that rat brain and rat embryo have more DLL3 protein than CNS-1 cells. By performing qRT-PCR with a GAPDH control, we found that when normal *Dll3* expression in rat brain RNA is normalized to 1.0, the expression of *Dll3* in CNS1 cells is only 0.003 fold of that in brain, showing that glioma cells have significantly lower endogenous *Dll3* expression than normal tissue.

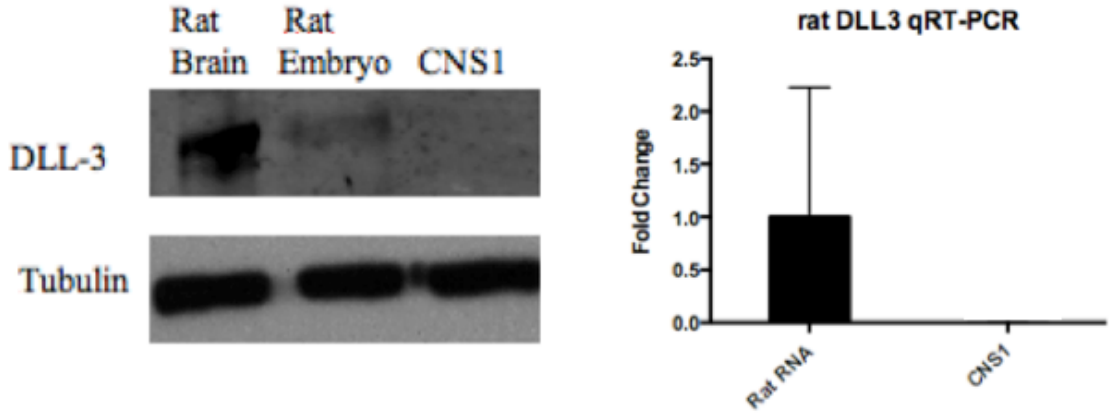

Figure 8. Endogenous DLL3 expression in the rat glioma cell line, CNS-1, was measured by both Western blot and qRT-PCR. When normalized to normal rat RNA, CNS1 *Dll3* mRNA levels are only 0.003 fold. In both cases, the DLL3 expression in the glioma cells was much lower than in normal rat brain, suggesting that this may contribute to why NICD is so highly expressed in CNS-1 cells.

CNS-1 cells are known to have high NICD activity (Kruse et al., 1994), so the results fit with the hypothesis that low *Dll3* expression means higher Notch activity and vice versa. Low expression of *Dll3*, a Notch inhibitor, in glioma cells could facilitate for overactive Notch pathway activity, contributing to the glioma stem cell phenotype. This finding also led to the conclusion that it would not be useful to complete experiments with *Dll3* siRNA in glioma cells as originally planned. As the endogenous *Dll3* in CNS-1 cells is so low, it is likely that no effect would be observed by knocking down *Dll3*, and thus only experiments where *Dll3* was overexpressed were conducted.

Overexpression of *Dll3* causes a decrease in CBF-1-controlled gene expression

To study the effect of DLL3 on Notch-1 activation, we performed Luciferase co-culture assays. Using a promoter that is activated by the CBF-1 transcription factor and a luciferase reporter gene, luciferase expression can be quantified and thus the activity of the promoter can be measured. CBF-1 is known to be activated by NICD, so as activation of Notch increases, the expression of luciferase will increase. Cells expressing both the reporter gene, renilla (transfection-efficiency control), and either pcDNA3 (control vector) or *Dll3* were co-cultured

with cells transfected with pBOS (control vector) or *Dll1* (an activator of the Notch receptor). The role of the *Dll1* cells was to deduce whether DLL3 could not only reduce the endogenous activity of Notch, but continue to repress Notch activity in the presence of a Notch activator.

When co-cultured with pBOS control cells, DLL3 lowered luciferase activity, and thus Notch-dependent CBF-1 activity ($p<0.001$*). This decrease remained even in the presence of DLL1 ($p=0.0056$*), suggesting that DLL3 is a potent inhibitor of Notch activity even when in the presence of overexpressed Notch activators.

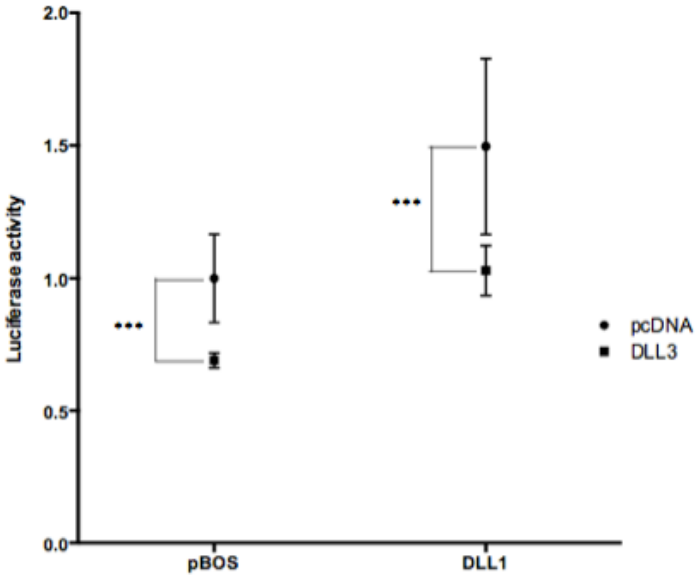

Figure 9. pcDNA or *Dll3* were transiently transfected into CNS-1 cells (stably transfected with a CBF-1 controlled lucierase gene) along with a renilla control by Lipofectamine transient transfection. The Luciferase/Renilla values were used for data analysis. DLL3 decreased promoter activity when co-cultured with a pBOS control ($p<0.001$*) and the Notch-activator DLL1 ($p=0.0056$*).

This data show us that DLL3 decreases Notch-dependent transcription, most likely by decreasing Notch-ligand interactions, which decrease Notch activation and transcription of Notch target genes. This suggests that other Notch targets under CBF-1 control, which are responsible for the glioma stem cell phenotype, would also be downregulated by exogenous *Dll3*, supporting the potential use of DLL3 as a glioma treatment.

Overexpression of *Dll3* does not change cell viability or proliferation

We proposed that cell viability will not be affected by *Dll3* expression, as the knockdown of Notch-1 has not been found to cause cell death or a change in metabolic activity. Cell viability was measured in an MTS assay, comparing CNS-1 cells transiently transfected with P16 (control vector) or *Dll3*. There is no statistical difference between the two groups (p-value for Day 7= 0.4750). This supports the hypothesis that a change in *Dll3* expression does not affect Notch-independent phenotypes; viability is not a phenotypic effect of the pathway and so remains unchanged. However, cells continue to proliferate throughout the assay, as the MTS absorbance increases; proliferation is Notch-dependent, thus we would expect to see *Dll3*-expressing cells stay at a relatively constant MTS absorbance as the cells don't proliferate but don't die either, while the control cells continue to grow.

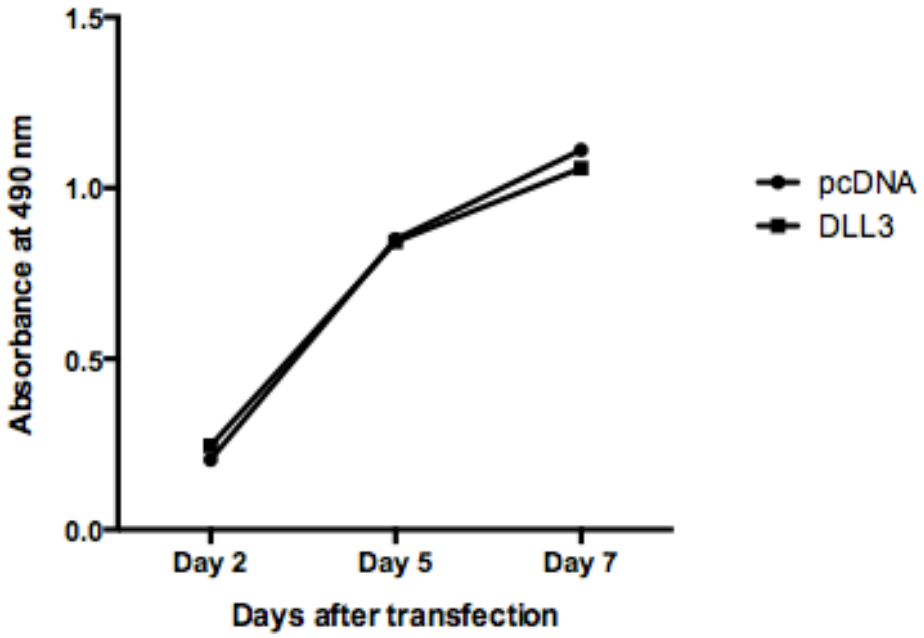

Figure 10. A MTS assay was performed to measure cell viability. CNS-1 cells were transiently transfected with *Dll3* or pcDNA, plated at 5,000 cells/well, then left to grow for 2-7 days. At different time points, the MTS reagant was added and absorbance at 490 nm was measured. No statistical significance was found (p-value for Day 7 =0.4750), which suggests there is no change in viability. However, we would expect proliferation to decrease in the *Dll3*-expressing cells, as it is Notch-dependent, however the proliferation remains equal between the two cell populations.

We had many issues with overexpressing and detecting *Dll3* in CNS-1 cells, so this may be due to insufficient *Dll3* expression, meaning we cannot extrapolate the viability data either. If we could more robustly express *Dll3*, we would expect to see decreased proliferation. To differentiate between viability and proliferation, we will need different assays as well, for example one that measures cell death, where we would expect to see no difference between the control and *Dll3* cells. We could also use flow cytometry to see how many cells are actively replicating, where we would expect to see fewer cells transfected with *Dll3* replicating.

It is important that viability is unchanged when *Dll3* is overexpressed because this suggests that DLL3 could be targeted as a treatment for glioma without affecting other cell functions. Unlike current chemotherapy treatments like TMZ, a DLL3 treatment may not kill other cells, which results in adverse effects. However, a distinction to be made in this experiment is that the MTS viability assay does not measure cellular life, but metabolic activity. The assay does not differentiate between quiescent cells and dead cells. This experiment does not tell us if cells are coming out of quiescence at all, or if cells are dying, only that the relative amount of metabolic activity is the same. In the future, it will be important to decipher whether DLL3 can affect cell quiescence, as this is a hallmark of glioma stem cells and one of the major difficulties with current treatments.

***Dll3* was not shown to affect chemoresistance**

The Notch pathway has been suggested as a possible source of chemoresistance in glioma, and upregulation of Notch-1 in glioma causes cells to become more resistant to the standard of care chemotherapy, TMZ (Purow et al., 2005). For this reason, we proposed that upregulation of *Dll3* would cause a decrease in chemoresistance of glioma cells. Cells were transfected with a P16 pcDNA control or *Dll3*, then exposed to either isovolumetric DMSO or

TMZ (final concentration of 400 μM). At various time points after exposure (Days 2, 4, and 6), cell viability was measured by MTS assay. There was no significant difference (p-value for Day 6= 0.2624) between P16 and DLL3 cells, suggesting *Dll3* expression does not change chemoresistance. However, there was no statistical difference between cells treated with DMSO and TMZ, meaning that treatment with TMZ did not work and wasn't killing cells.

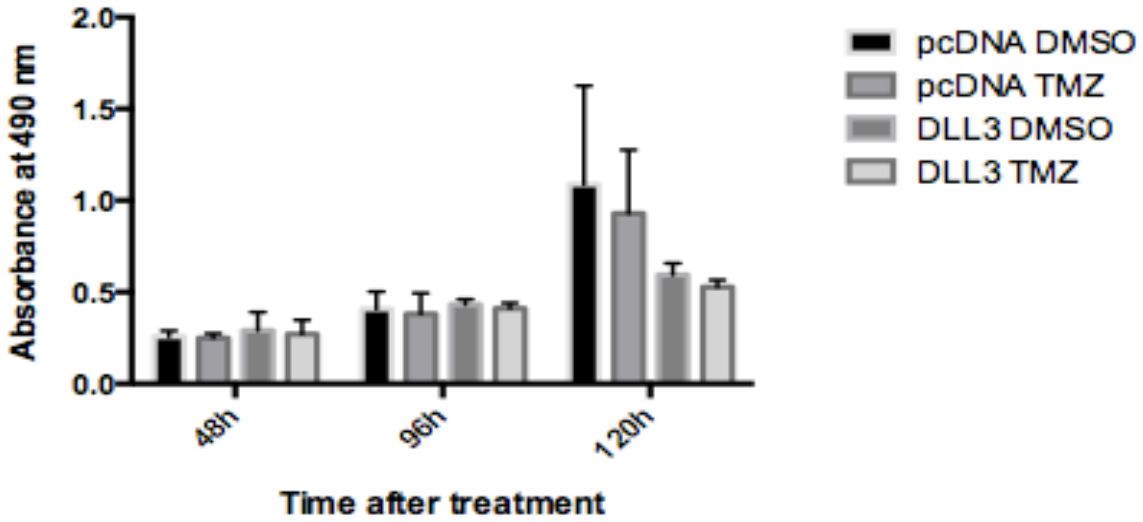

Figure 11. CNS-1 cells were transiently transfected with *Dll3* or P16 control, plated at 5,000 cells/well, then exposed to 400 micromolar TMZ or isovolumetric DMSO 24 hours later. Cells were left to grow for 2-6 days. At different time points, a MTS assay was performed and absorbance at 490 nm was measured. No statistically significant difference was found between P16 and *Dll3*-expressing cells (p-value for Day 6= 0.2624). However, cells in TMZ appear to have similar survival to cells in the DMSO control, suggesting that our TMZ treatment did not work.

It is very important to determine whether *Dll3* expression affects chemoresistance, as this is one of the major issues with current glioma treatment that we are hoping to address. While these results are not supportive of our hypothesis, we will need to optimize our TMZ treatments before we can draw conclusions about the role of DLL3 in chemoresistance. It is possible that our CNS-1 cell line acquired resistance to TMZ, or that the treatment protocol was not being followed correctly. Until our results can show statistically significant drug treatment similar to other published results, we cannot determine whether *Dll3* overexpression affects chemoresistance.

Discussion

The Notch pathway has a complex mechanism of action

Through our studies on double mutants' axial skeletal development, we found that when both *Dll3* and *Lfng* are knocked out, the phenotype is worse than a mutation in just one of the genes. We previously believed that the double mutants would resemble *Dll3* pudgy mutants, but it is now clear that LFNG and DLL3 act differently throughout the PSM to influence development. This shows that the Notch pathway is not a linear pathway throughout the entire PSM; if it were, the inhibition of DLL3 activity would result in the same phenotype as the double mutants. Instead, we found that in the posterior PSM, where the clock is active, *Dll3* is epistatic to *Lfng*, while in the anterior PSM, they act together in parallel pathways. Very little research has been done on these two modulators together, and investigation of the Notch pathway is complicated by the numerous ligands that activate/inhibit the receptor.

In our immunohistochemistry experiments, we found that *Lfng*-null embryos have activated Notch throughout the PSM, and inhibition of *Dll3* causes a decrease in Notch activity. This runs counter to the canonical knowledge of LFNG as an activator of Notch and DLL3 as an inhibitor. When an activator is knocked out, activity is expected to decrease, and vice versa for an inhibitor. Why this occurs is not clear, but it appears that our understanding of Notch and the role it plays in different contexts is not complete.

We also found that while mice with either *Dll3*-pudgy or *Lfng*-null mutations have formed neural arches (the posterior part of the vertebra), double mutants do not. This implies that there is a rescue-of-function in single mutation mice, and that neural arch development can occur with either *Lfng* or *Dll3* present. This is surprising, as the two have very different functions,

LFNG as a Notch activator, and DLL3 as an inhibitor. Why the other ligands that are present in the pathway cannot rescue the development of neural arches in double mutants is also not clear.

The *Dll3* phenotype is epistatic to the *Lfng* phenotype in the segmentation clock

Through our use of immunohistochemistry, we found that 10.5 d.p.c. mouse embryos with both *Lfng* and *Dll3* mutations have a similar Notch activation pattern to *Dll3* pudgy embryos in the posterior PSM, where the clock is active. This suggests that *Dll3* is epistatic to *Lfng*, meaning that LFNG activity depends on the presence of DLL3 or that DLL3 activity occurs first in the pathway. This also shows that the two modulators interact with the Notch receptor through a similar mechanism, which is why the double mutants resemble the pudgy mutants instead of a mixture of the two single mutants. By showing that DLL3 activity can influence LFNG activity, we can utilize DLL3 in both CNS development and glioma when trying to downregulate Notch, because LFNG will not be able to negate DLL3's effects.

However, this relationship is not epistatic in the anterior PSM, where somites are patterned and the clock is no longer active. The bands of activation in double mutants were more faint than in the *Dll3*-pudgy mutants. Why this occurred, as opposed to an upregulation in the posterior PSM like the *Lfng* mutants, is not clear. While these results show us that *Dll3* is sometimes epistatic to *Lfng*, to fully understand the DLL3/LFNG relationship, we will need to do further experiments to explain the mechanism behind this relationship.

The Notch pathway is an important glioma treatment target

Through both Western blotting and qRT-PCR, we saw that DLL3 expression is much lower in the rat glioma cell line, CNS-1, than in normal rat brain. This, along with the fact that CNS-1 cells have been shown to have high NICD expression (Kruse et al., 1994), suggests that low DLL3 expression means high Notch activity. Thus, by upregulating *Dll3*, we hypothesized

we could decrease Notch activity. These results also led us to the decision to not attempt knocking down *Dll3* in CNS-1 cells as a comparison to upregulation, as the effects of knockdown would be very small.

The recent clinical success of γ-secretase inhibitors in glioma also suggests that inhibition of Notch activity can better patient outcomes. However, there are some drawbacks to γ-secretase inhibitor treatment. Notch signaling is active throughout the body, so inhibiting the pathway could have many side effects. For example, because Notch signaling is critical for normal intestinal differentiation, γ-secretase inhibitors cause progenitor cells to differentiate into goblet cells, interfering with the intestinal epithelium, which is already sensitive due to chemotherapy. Besides cleaving the NICD fragment, γ-secretase plays other roles in the body, including the cleavage of amyloid precursor protein (APP). The drug does not differentiate between the different roles of γ-secretase, and so will interfere in other pathways as well. γ-secretase inhibitors can also target similar proteins, like α- and β-secretase, which have a variety of functions (reviewed in Shih and Wang, 2007). This makes these drugs' long-term effects hard to predict, and gives reason to pause when considering them as a therapeutic option.

DLL3 is a promising inhibitor of the Notch pathway in glioma

Using Luciferase co-culture assays, we found that overexpression of *Dll3* does decrease Notch target activity. While this does not actually measure the activity of NICD, it focuses on the transcription of target genes, which is the main way that Notch affects phenotype. By showing that *Dll3* overexpression does not affect Notch-independent phenotypes, like viability, we can be hopeful that DLL3 would have fewer side effects than current chemotherapy treatments, like TMZ, which targets all replicating cells. Instead, DLL3 treatment could leave normal cells alone and allow for patients to have fewer side effects than conventional chemotherapy. While we did

not see the expected changes in proliferation, we believe this is because of transfection errors that did not allow us to reach the necessary amount of *Dll3* expression.

Our chemoresistant assays did not generate any helpful data because of issues with the TMZ treatment. The TMZ did not kill the cells any more than the DMSO control (which was isovolumetric to the DMSO used to solubilize the TMZ). This could be because of a problem with the TMZ compound itself, the dosage, or the DMSO; the cells could also have developed resistance to TMZ after extended periods of being in culture. We will need to spend more time optimizing TMZ treatment and experimenting with other cell lines to determine the source of the problem. Nevertheless, these results should not deter further exploration of DLL3 and its role in chemoresistance.

Another point of interest is that Lunatic Fringe, an activator of the Notch pathway, is relatively upregulated in glioma with poorer outcomes. Glioma patients with underexpressed Lunatic Fringe have a better survival (p-value= 0.04 when compared to intermediate Lfng expression). This suggests that LFNG activation plays a role in Notch's hyperactivity in glioma and contributes to a GSC phenotype that worsens survival. Our epistasis results give hope that by upregulating *Dll3*, we could negate the activating effects of LFNG in glioma. Very little research has been done on LFNG in glioma, and by focusing on DLL3's ability to overpower activators of the Notch pathway, like LFNG, we can begin to understand more of the mechanism of this complex relationship.

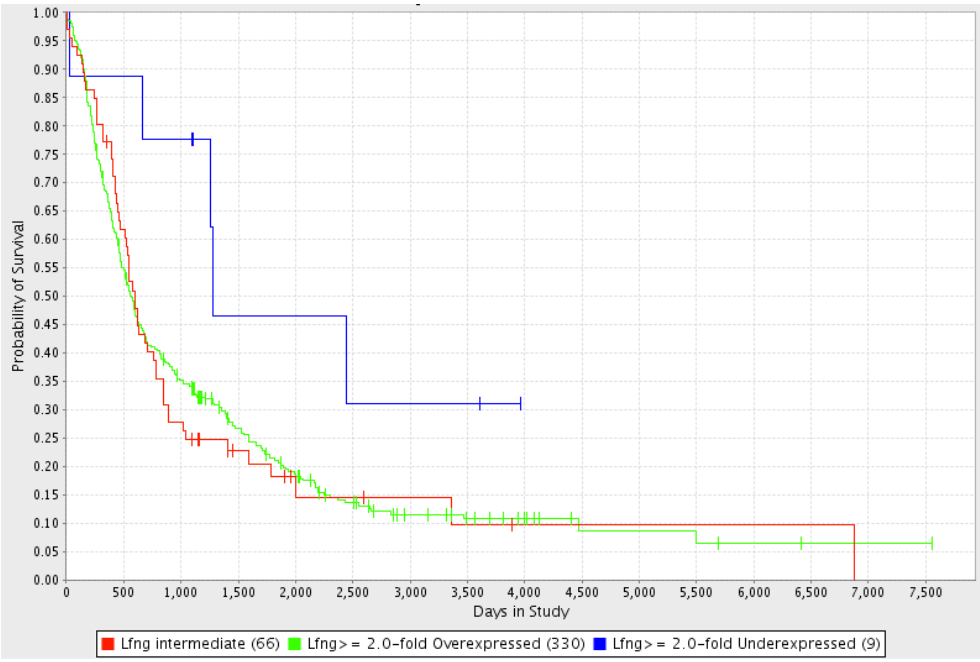

Figure 12. Kaplan-Meier survival plot of gliomas with different Lunatic Fringe expression levels. Underexpressed Lunatic Fringe (2x below the mean expression) has a better survival outcome than both intermediate Lunatic Fringe expression (p-value=0.04) and overexpressed Lunatic Fringe (p-value=0.05). This suggests that Lunatic Fringe may be involved in glioma development and metastasis. However, there is no statistical difference in survival between gliomas with intermediate and overexpressed Lunatic Fringe (p-value=0.84). Data from REMBRANDT (National Cancer Institute).

Future Directions

In our studies of segmentation, further research on how DLL3 and LFNG interact should be completed. In particular, looking at the effects of the *Dll3*-pudgy/*Lfng*-null double mutations on *Hes7* expression in the PSM would reveal if there are further changes in the downstream pathway from mutations in either gene. The use of mouse models that constitutively overexpress *Dll3* and *Lfng* could act as a useful supplement to studying how the two ligands interact.

Moving forward with our glioma research, we should focus on GSC phenotypes, like chemoresistance and regeneration of heterogeneous tumors, when *Dll3* is overexpressed. This would give more evidence as to whether our hypothesis that DLL3 increases survival by decreasing GSCs is correct. To do this, we must optimize our chemotherapy treatment, as we

struggled with treating CNS-1 cells with TMZ. While the use of TMZ is ideal, as it is the current standard of care for glioma, a different glioma cell line might be more susceptible to treatment. Another way to study DLL3's role in GSCs is to use a GSC line, or a cell line where the proportion of CD133+ cells is high. While the overall effect of overexpressing *Dll3* may be small when the percentage of CD133+ cells is low, by studying CD133+ cells only we can study DLL3's specific effects on GSCs.

Based on the results from future experiments, we may want to pursue the creation of stable cell lines with overexpressed/knockdown *Dll3*. These cells could be implanted intracranially in mice to analyze tumor development and progression compared to control glioma cells. Another exciting direction of this project would be to explore possible therapeutic strategies targeting DLL3. DLL3 itself could be administered intravenously to mice with glioma tumors. If DLL3 is not a viable therapeutic option itself, exploration into possible activators of DLL3 would need to be started. DLL3 appears to be a promising inhibitor of Notch activity in glioma cells, suggesting it could be targeted in future treatment options. If DLL3 can be used as a target for future Notch inhibition in glioma, we may be able to make gliomas more susceptible to current treatments and improve patient outcomes.

Acknowledgments

Firstly, I would like to thank my advisor, Dr. Susan Cole, for her support and mentorship throughout this process. Switching labs during my honors thesis was a difficult adjustment, but Dr. Cole helped me work smoothly with excellent guidance while allowing me the independence I needed to learn as the project continually developed. I would also like to thank Dr. Mariano Viapiano for encouraging my interest in the scientific process, designing the initial project, and being a continuing source of mentorship and collaboration. I would like to thank Dr. Pawan Kumar and Dr. Margaret Teaford for dedicating their time and expertise to help me in my continual quest to become a better researcher, as well as being flexible committee members with the many changes this project has undergone. I would like to thank Kanu Wahi for her help and for advising me, as well as mentoring me in the lab. I would also like to thank the rest of the Cole lab- M. Skye Bochter, Kara Braunreiter, Antony Miller, and Dustin Williams, for their patience and dedication. Thank you to the Undergraduate Research Office and the Pelotonia Undergraduate Fellowship for supporting me financially as I continued to work on this project.

References

Beier D, Rohri S, Pillai DR, et al. Temozolomide preferentially depletes cancer stem cells in glioblastoma. *Cancer Res* **2008**; *68* (14): 5706-5715.

Bessho Y, Sakata R, Komatsu S, et al. Dynamic expression and essential functions of hes7 in somite segmentation. *Genes Dev* **2001**; *15* (20): 2642-2647.

Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **2006**; *7* (9): 678-689.

Brucker K, Perez L, Clausen H, et al. Glycosyltransferase activity of Fringe modulates Notch-Delta interations. *Nature* **2000**; *406* (6794): 411-415.

Christ B, Jacob HF, Jacob M. Somitogenesis in the chick embryo [Determination of the segmentation direction]. *Verh Anat Ges* **1974**; *68*: 573-579.

Cooke J, Zeeman EC. A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *J Theor Biol* **1976**; *58* (2): 455-476.

Dell'Albani P. Stem Cell Markers in Gliomas. *Neurochem Res* **2008**: *33*: 2407-2415.

D'Souza B, Meioty-Kapella L, Weinmaster G. Canonical and non-canonical Notch ligands. *Curr Top Dev Biol* **2010**; *92*: 73-129.

Evrard YA, Lun Y, Aulehla A, et al. Lunatic fringe is an essential mediator of somite segmentation and patterning. *Nature* **1998**; *394* (6691): 377-381.

Fan X, Matsui W, Khaki L, et al. Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. *Cancer Res* **2006**; *66* (15): 7445- 7452.

Geffers I, Serth K, Chapman G, et al. Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. *J Cell Biol* **2007**; *178* (3): 465-476.

Goldthwaite CA. Are Stem Cells Involved in Cancer? *Regenerative Medicine.* Department of Health and Human Services. August 2006. </info/scireport/2006report.htm>

Gridley T. Notch signaling and inherited disease syndromes. *Hum Mol Genet* **2003**; *12*: R9-R13.

Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* **2005**; *351* (10): 997-1003.

Hirata H, Bessho Y, Kokubu H, et al. Instability of Hes7 protein is crucial for the somite segmentation clock. *Nat Genet* **2004**; *36* (7): 750-754.

Hu B, Nandhu MS, Sim H, et al. Fibulin-3 Promotes Glioma Growth and Resistance through a Novel Paracrine Regulation of Notch Signaling. *Cancer Res* **2012**; *72* (15): 3873-3885.

Ignatova TN, Kukekov VG, Laywell ED, et al. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* **2002**; *39*: 193-206.

Kanu OO, Mehta A, Di C, Lin N, Bortoff K, Bigner DD, Yan H, Adamson DC. Glioblastoma multiforme: a review of therapeutic targets. *Expert Opin Ther Target* **2009**; *13* (6): 701-718.

Koch U, Radtke F. Notch and cancer: a double-edged sword. *Cell Mol Life Sci* **2007**; *64*: 2746-2762.

Krop I, Demuth T, Guthrie T, et al. Phase I pharmacologic and pharmacodynamic study of the gamma secretase (Notch) inhibitor MK-0752 in adult patients with advanced solid tumors. *J Clin Oncol* **2012**; *30* (19): 2307-2313.

Kruse CA, Molleston MC, Parks ET, et al. A rat glioma model, CNS-1, with invasive characteristics similar to those of human gliomas: a comparison to 9L gliosarcoma. *J Neurooncol* **1994**; *22* (3): 191-200.

Kusumi K, Sun ES, Kerrebrock AW, et al. The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. *Nat Genet* **1998**; *19* (3): 274-278.

Ladi E, Nichols JT, Ge W, et al. The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. J *Cell Biol* **2005**; *170* (6): 983-992.

Lasky JL, Wu H. Notch signaling, brain development, and human disease. *Pediatr Res* **2005**; *5*: 104-109.

Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* **2006**; *5*.

Mrugala MM, Chamberlain MC, Hutchinson F. Mechanisms of disease: temozolomide and glioblastoma—look to the future. *Nature Clinical Practice Oncology* **2008**; *5*(8): 476–486.

National Cancer Institute. 2005. Kaplan-Meier survival plot for Gene Expression, search: Dll3. REMBRANDT. <http://rembrandt.nci.nih.gov>. Accessed 2012 December 8.

National Cancer Institute. 2005. Kaplan-Meier survival plot for Gene Expression, search: Lfng. REMBRANDT. <http://rembrandt.nci.nih.gov>. Accessed 2014 February 12.

Natsume A, Kinjo S, Yuki K, et al. Glioma-initiating cells and molecular pathology: implications for therapy. *Brain Tumor Pathol* **2011**; *28*: 1-12.

Palmeirim I, Henrique D, Ish-Horowicz D, et al. Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* **1997**; *91* (5): 639-648.

Philips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* **2006**; *9* (3): 157-173.

Purow BW, Haque RM, Noel MW, et al. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res* **2005**; *65*: 2353-2363.

Sanderson BJS, Shield AJ. Mutagenic damage to mammalian cells by therapeutic alkylating agents. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1996**; 355: 41-57.

Shifley ET, Vanhorn KM, Perez-Balaguer A, et al. Oscillatory lunatic fringe activity is crucial for segmentation of the anterior but not posterior skeleton. *Development* **2008**; *135* (5): 899-908.

Shih I, Wang T. Notch Signaling, γ-Secretase Inhibitors, and Cancer Therapy. *Cancer Res* **2007**; *67* (5): 1879-1882.

Truett GE, Heeger P, Mynatt RL, et al. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* **2000**; *29* (1): 52-54.

Vik-Mo EO, Sandberg C, Joel M, et al. A comparative study of the structural organization of spheres derived from the adult human subventricular zone and glioblastoma biopsies. *Exp Cell Res* **2011**; *317*: 1049-1059.

Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med* **2008**; *359* (5): 492- 507).

Zhang XP, Zheng G, Zou L, et al. Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. *Mol Cell Biochem* **2008**; *58*: 101-108.